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PEPTIDE DERIVATIVE AND ITS USE

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[There are no amendments to this patent.]

Abstract

Constitution

Peptide derivatives having certain structure, antioxidants containing such peptide derivatives as effective components, and antimicrobial agents containing such peptide derivatives as effective components.

Effects

Antioxidants having excellent suppression actions against active oxygen, free radicals and lipid peroxidation and antimicrobial agents having excellent antimicrobial actions against microorganisms such as bacteria, fungi, etc., are provided Such antioxidants and antimicrobial agents are extremely safe when used in pharmaceuticals, foods, etc.

Claims

1. Peptide derivative represented by the general formula a), b), or c):

a) R_1-X

b) X-R_2

c) R_1-X-R_2

(In the above chemical formulas, R_1 represents an acetyl group, acyl group, or polyethylene glycol; X is a peptide having an amino acid sequence described in any of the sequence numbers 1-3 of amino acid in D or L form; R_2 represents an amino group, acyl group, or polyethylene glycol).

2. Antioxidant containing, as effective component, a peptide derivative represented by the general formula a), b), or c):

a) R_1-X

b) X-R_2

c) R_1-X-R_2

(In the above chemical formulas, R_1 represents an acetyl group, acyl group, or polyethylene glycol; X is a peptide having an amino acid sequence described in any of the sequence numbers 1-3 of amino acid in D or L form; R_2 represents an amino group, acyl group or polyethylene glycol).

3. Antioxidant according to Claim 2, with a peptide derivative content of at least 1 μg/mL.

4. Antimicrobial agent containing, as effective component, a peptide derivative represented by the general formula a), b), or c):

a) R_1-X

b) X-R_2

c) R_1-X-R_2

(In the above chemical formulas, R_1 represents an acetyl group, acyl group, or polyethylene glycol; X is a peptide having an amino acid sequence described in any of the sequence numbers 1-3 of amino acid in D or L form; R_2 represents an amino group, acyl group, or polyethylene glycol).

5. Antimicrobial agent according to Claim 4, with a peptide derivative content of at least 1 μg/mL.

Detailed explanation of the invention

[0001]

Industrial application field

The present invention concerns peptide derivatives and uses thereof, more specifically safe peptide derivatives that can be used in pharmaceuticals and other products.

[0002]

Prior art

Recently, free radical participation has been tied to the conditions of various ailments. We need oxygen for maintaining life, while in the body, active oxygen and free radicals are continuously formed, and free radicals once formed can cause modification of membrane lipids and membrane proteins, damaging the cell membranes and causing various ailments by altering DNA and enzymes. The highly unsaturated fatty acids in the cell membranes are especially easily attacked by free radicals, resulting in formation of harmful peroxidized lipids, leading to increased cell damage (Gendai Iryo, Vol. 25, p.163, 1993).

[0003]

The active oxygen-related ailments are present in a wide range of burns, inflammation such as arthritis, reflux disorder, side reaction of anticancer agents, radiation disorder, gastric ulcer, microbial shock, cachexia, autoimmune disorder, etc. Thus, it is considered that suppression of active oxygen, free radicals, and peroxidized lipids is effective for prevention and treatment of such ailments (Nikkei Biotech ed., "Nikkei Bio Yearbook," p. 350, Nikkei BP, 1994).

[0004]

While many antimicrobial agents have been developed and used, infections have not decreased, and causes and symptoms have become very complex. An increase in drug resistance

of infectious bacteria, appearance of compromised host, etc., make a solution especially difficult, thus new prevention and treatment methods of infections are desired.

[0005]

One of them [antimicrobial agents] is the lactoferrin. This is an iron-bonded protein present in tears, saliva, milk, neutrophilic granules, etc. and known to have diverse physiological activities, such as antimicrobial action, antioxidant action, immune control action, etc. (Archives of Disease in Childhood, Vol. 67, p. 657, 1992). It is also known that lactoferrin decomposition products, lactoferrin-based peptides, homological peptides also have antimicrobial and antioxidant actions, e.g., antimicrobial agents using lactoferrin decomposition products as the effective component (Japanese Kokai Patent Application No. Hei 5[1993]-320068), antimicrobial peptides having at least 20 amino acid moieties (Japanese Kokai Patent Application No. Hei 5[1993]- 92994), antimicrobial peptides from 11 amino acid residues (Japanese Kokai Patent Application No. Hei 5[1993]-78392), antimicrobial peptides from 6 amino acid moieties (Japanese Kokai Patent Application No. Hei 5[1993]-148297), antimicrobial peptide from 5 amino acid moieties (Hei 5[1993]-148296), antimicrobial peptides having 3-6 amino acid moieties (Japanese Kokai Patent Application No. Hei 5[1993]-148295), antioxidants containing, as effective components, lactoferrin decomposition products, peptides separated from lactoferrin decomposition products, and synthetic peptides having the same amino acid sequence as that of peptide separated from the lactoferrin decomposition products (Japanese Kokai Patent Application No. Hei 6[1994]-199687), etc.

[0006]

For the peptide derivatives, various studies are carried out for utilizing the properties not found in simple peptides. Peptides are derivatized for increased activity, imparting resistance against protease, increased in vivo retention, etc., e.g., metal peptides having antioxidant activity and inflammation resistance (Japanese Tokuhyo Patent Application No. Hei 5[1993]-503939), tripeptide arginal derivatives suppressing the ischemia and re-perfusion injuries (Japanese Kokai Patent Application No. Hei 6[1994]-199682), quaternary ammonium peptides used for skin cosmetics (Japanese Kokai Patent Application No. Hei 4[1992]-82822), N-substituted peptides of amphiphilic ion channel-forming peptides having biological activities against cell growth, virus, virus-infected cells, etc. (WO93/24138), C-terminal substituted peptides of amphiphilic ion channel forming peptides having biological activities (WO92/22317), etc.

[0007]

Problems to be solved by the invention

However, for the lactoferrin peptides, physiological activities of derivatives such as antioxidant properties, antimicrobial properties, etc., have not been known, thus there are no antioxidants and antimicrobial agents based on those peptide derivatives as effective components. With such prior art in hand, it is an objective of the present invention to provide safe antioxidants and antimicrobial agents using lactoferrin peptide derivatives as effective components, entirely different from conventional antioxidants and antimicrobial agents.

[0008]

Means to solve the problems

The present invention solves the problems described above and provides antioxidants and antimicrobial agents using lactoferrin peptide derivatives of certain chemical formulas or as the effective components. Next, the present invention is explained in detail.

[0009]

The peptide derivatives used in the present invention can be prepared by a method for obtaining the desired peptides by synthesis using a known method (e.g., peptide automatic synthesizer); method of adding an acyl group, polyethylene glycol, etc., by organic synthesis technology, to peptides synthesized by using a peptide synthesizer; method involving hydrolysis of lectoferrins by an acid or enzyme, followed by fractionation of peptides with certain amino acid sequence from the hydrolysis mixture by a separation means, such as liquid chromatography, etc., then adding acyl group, polyethylene glycol, etc., by using organic synthesis technology.

[0010]

Next, as examples of peptide derivatives of the present invention, preparation methods for peptides having acetyl group at the terminal amino group and amide group at the terminal carboxy group are illustrated. The preparation is performed by solid-phase peptide synthesis method by Shepherd, et al., Journal of Chemical Society Perkin I, p. 538, 1981, using a peptide synthesizer (LKB Biolynx 4170, product of Pharmacia LKB Biotechnology Co.).

[0011]

Using a solid resin for C-terminal amide peptide synthesis (NovaSyn KR 0.1 mEq, product of Cal Biochem-Nova Biochem Co.) and the synthesis program of the above peptide synthesizer, the protective group removal reaction and condensation reaction are repeated for

peptide chain extension. Namely, by 20% piperidine/dimethylformamide (hereafter referred to as DMF), the amino protective group 9-fluorenylmethoxycarbonyl (hereafter referred to as Fmoc) group is cleaved and removed, followed by washing with DMF, Fmoc-amino acid active ester/N-hydroxybenzotriazole (hereafter referred to as HOBt) reaction, washing with DMF, repeating the process.

[0012]

After the condensation, if needed, a Kyser test is carried out to confirm the completion of coupling before proceeding to the next step. The amino acids used are Fmoc-Arg(Mtr)-Opfp, Fmoc-Lys(Boc)-Opfp, Fmoc-Trp(Boc)-Opfp, Fmoc-Gln-Opfp, Fmoc-Met-Opfp (all products of Cal Biochem-Nova Biochem Co.) in 0.5 mmol cartridges. After completion of peptide chain extension reaction, Fmoc group is cleaved by 20% piperidine/DMF, followed by washing with DMF then acetylation by 10% acetic anhydride/DMF. After confirmation of completion of the acetylation by the Kyser test, the resin is then washed well with DMF, tert-pentyl alcohol, acetic acid tert-pentyl alcohol, DMF, then diethyl ether and dried in vacuo.

[0013]

The above protected peptide resin is stirred with ethanedithiol and thioanisole in an argon stream at room temperature, further stirred under ice cooling, stirred with trifluoroacetic acid, then with trimethylsilyl bromide. The resin is filtered out using a glass filter, and the filtrate is concentrated in vacuo immediately. The residue is treated with cold diethyl ether to form a white peptide powder which is then separated by centrifugal separation, freed from the supernatant, stirred with cold diethyl ether again, and subjected to centrifugal separation. This separation process is repeated four times. The peptide precipitate is dried in vacuo, dissolved in water and lyophilized to obtain a crude peptide.

[0014]

The crude peptide is dissolved in water and centrifuged. The supernatant is filtered through a 0.45 mm filter, and the peptide is purified by high-performance liquid chromatography (HPLC). The HPLC is conducted using Gariba PU-986 high-pressure gradient system (product of Nippon Bunko Co.) and reverse-phase column Lichrospher 100RP-18(e) 250 x 10 mm (product of Merck Co.) Elution is performed with a solution A of 0.1% TFA/water and a solution B of 80% acetonitrile/solution A, with a solution A to solution B linear concentration gradient. The chromatogram almost has a single peak. The corresponding fraction is separated repeatedly and lyophilized to obtain a purified peptide.

[0015]

The purified product is confirmed to be a single compound by HPLC analysis using an analytical column (Lichrospher 100RP-18(e) 250 x 4.6 mm, product of Merck Co.) The purified peptide structure is confirmed by conventional amino acid analysis, amino acid sequence analysis, elemental analysis, mass analysis, etc. The addition of polyethylene glycol, acyl group, etc., to the peptide obtained by lactoferrin hydrolysis, etc., is illustrated below.

[0016]

Acetic anhydride is reacted with $CH_3(OCH_2CH_2)_nOH$ (monomethoxypolyethylene glycol (hereafter referred to as mPEG), product of Polyscience Co.) to obtain mPEG-COOH which is then treated with N-hydroxysuccinimide and dicyclohexylcarbodiimide to synthesize mPEG N-hydroxysuccinimide ester. A lactoferrin peptide fragment is treated with the mPEG succinimide ester to obtain a polyethylene glycol-modified product. Also, similarly, a fatty acid succinimide ester is prepared from $CH_3(CH_3)_n$-COOH [sic] then reacted with a lactoferrin peptide fragment to obtain an acylation product. There are no particular restrictions in the molecular weight of the polyethylene glycol and acyl group.

[0017]

The peptide derivatives of the present invention can be used not only as drugs, but also in products taken into the body by humans and animals such as food, feeds, cosmetics, etc., or skin application or compounded in the products used for suppression of active oxygen, free radicals, and lipid peroxidation, and suppression and sterilization of microorganisms such as bacteria, fungi, etc. Also, products and raw materials can be treated with the peptide derivatives of the present invention. Namely, the peptide derivatives of the present invention can be administered as is to humans or animals or used in drugs (e.g., preventive drugs for various ailments, treatment drugs for various ailments, antimicrobial agents, etc.), food (e.g., powdered milk, nutrients, chewing gums, etc.), nondrug products (e.g., mouthwash, health drinks, etc.), various cosmetics (e.g., creams, emulsions, sunscreen products, skin aging preventives, etc.), raw materials thereof, products used for suppression of active oxygen, free radicals, and lipid peroxidation, and suppression and sterilization of microorganisms such as bacteria, fungi, etc., by addition, compounding, spray, coating, impregnation, etc., or treatment of the products used for suppression of active oxygen, free radicals, and lipid peroxidation, and suppression and sterilization of microorganisms such as bacteria, fungi, etc. They can also be used together with known antioxidants and antimicrobial agents.

[0018]

Next, the present invention is explained in further detail with experimental examples.

Experimental Example 1

1) Preparation of samples

Next, eight samples were prepared.

Sample 1: Peptide with sequence number 4 prepared by the method described in reference example by enzymolysis of bovine lactoferrin

Sample 2: Peptide derivative of the Structure 1 below prepared by the method described in Application Example 1 by acetylation of the terminal amino group and amidation of the terminal carboxy group

[0019]

[Structure 1]

CH_3CO-Lys Lys Trp Gln Trp Lys Met Lys Lys-NH_2

5 a.a. cationic
no glu or Asp
+5
R1 = C1 alkyl

[0020]

Sample 3: Peptide derivative of the Structure 2 below prepared by the method described in Application Example 2 by bonding an acyl group of 18 carbon atoms to the terminal amino group and amidation of the terminal carboxy group

[0021]

[Structure 2]

Acyl(C18)-Arg Arg Trp Gln Trp Arg Met Lys Lys-NH_2

[0022]

Sample 4: Peptide derivative of the Structure 3 below prepared by the method described in Application Example 3 by bonding an acyl group of 16 carbon atoms to the terminal amino group and amidation of the terminal carboxy group

[0023]

[Structure 3]

Acyl(C16)-Arg Arg Trp Gln Trp Arg Met Lys Lys-NH_2

[0024]
Sample 5: Peptide derivative of the Structure 4 below prepared by the method described in Application Example 4 by bonding an acyl group of 14 carbon atoms to the terminal amino group and amidation of the terminal carboxy group

[0025]
[Structure 4]

Acyl(C14)-Arg Arg Trp Gln Trp Arg Met Lys Lys-NH_2

[0026]
Sample 6: Peptide derivative of the Structure 5 below prepared by the method described in Application Example 5 by bonding an acyl group of 10 carbon atoms to the terminal amino group and amidation of the terminal carboxy group

[0027]
[Structure 5]

Acyl(C10)-Arg Arg Trp Gln Trp Arg Met Lys Lys-NH_2

[0028]
Sample 7: Peptide derivative of the Structure 6 below prepared by the method described in Application Example 6 by bonding an acyl group of 6 carbon atoms to the terminal amino group and amidation of the terminal carboxy group

[0029]
[Structure 6]

Acyl(C6)-Arg Arg Trp Gln Trp Arg Met Lys Lys-NH_2

[0030]
Sample 8: Peptide derivative of the Structure 7 below prepared by the method described in Application Example 7 by acetylation of the terminal amino group and bonding polyethylene glycol (hereafter referred to as PEG) to the terminal carboxy group

[0031]
[Structure 7]

CH_3CO-Arg Arg Trp Gln Trp Arg Met Lys Lys-PEG

[0032]

2) Testing method

The Gutelidge [transliteration] method (Analytical Biochemistry, Vol. 82, p. 76, 1977) was partially modified and used for testing the antioxidant effects of each sample. In a pear-shaped flask, 20 mL 10 mM HEPES buffer (pH 7.4), 100 mg egg yolk phospholipid (product of Nakaritex Co.) and glass beads were added, followed by nitrogen purging, stirring at 37°C for 5 min using a Boltex mixer, keeping the mixture at 4°C for 1 h, and the supernatant was used as a liposome solution.

[0033]

In a 10-mL plastic tube, 0.5 mL of the liposome solution was treated with 100 μL sample solution of 7-fold concentration, 50 μL 112 μM $FeNH_4(SO_4)_2 \cdot 12H_2O$ (product of Nakaraitex Co.) and 50 μL 532 μM ascorbic acid (product of Nakaraitex Co.), the resulting mixture was stirred for 5 sec, and the tube was capped and incubated at 37°C for 1 h. 3 mL 1% sodium arsenite (product of Wako Junyaku Co.) solution in 2.9M HCl has added, mixed, and [the mixture was] centrifuged at 4000 rpm for 15 min. The clear solution portion (3 mL) was removed, treated with 1 mL 1% thiobarbituric acid (product of Nakaraitex Co.) solution in 0.05M NaOH, heated in boiling water for 15 min, allowed to cool and filtered through a 0.45 μM filter (product of Advantek Co.) The filtrate was measured for absorbance at 532 nm to obtain the degree of peroxidation of the liposome phospholipid. A measurement was also made for comparison with 100 μL distilled water added in place of the sample solution.

[0034]

The oxidation suppression (%) of the sample was calculated using the equation below.
Oxidation suppression = (comparison absorbance - sample absorbance)/comparison absorbance) x 100

3) Test results

The test results are given in Table 1. As clearly shown in Table 1, at a concentration of 100 μg/mL, Samples 2-8 showed stronger oxidation suppression than Sample 1. Samples 3-7 and Samples 3-6 showed oxidation suppression even at low concentrations of 10 and 1 μg/mL, respectively. When similar tests were made for other peptide derivatives, nearly similar results were obtained.

[0035]

Table 1

1 ペプチドの種類	2 濃度（μg/ml）による抑制率（%）			
	1000	100	10	1
3 試料 1	73.9	0.5	–	–
試料 2	71.7	12.6	–	–
試料 3	85.4	88.7	75.7	16.2
試料 4	87.9	89.6	62.7	9.0
試料 5	87.9	90.2	59.2	8.0
試料 6	91.9	86.7	59.2	4.0
試料 7	87.3	54.9	11.9	–
試料 8	72.6	20.9	–	–

Key: 1 Type of peptide
2 Suppression (%) by concentration (μg/mL)
3 Sample

[0036]

Experimental Example 2

This test is for the antimicrobial action by various compounds.

1) Preparation of samples

Eight samples were prepared as shown below.

Sample 1: Same as Sample 1 of Experimental Example 1

Sample 2: Peptide derivative of the Structure 8 below prepared by the method described in Application Example 8 by amidation of the terminal carboxy group.

[0037]

[Structure 8]

Lys Lys Trp Gln Trp Lys Met Lys Lys-NH_2

[0038]

Sample 3: Peptide derivative of the Structure 9 below prepared by the method described in Application Example 9 by bonding an acetyl group to the terminal amino group and amidation of the terminal carboxy group, with all of the amino acids being in D form.

[0039]

[Structure 9]

CH_3CO-Arg Arg Trp Gln Trp Arg Met Lys Lys-NH_2

[0040]

Sample 4: Same as Sample 3 of Experimental Example 1.

Sample 5: Same as Sample 4 of Experimental Example 1.

Sample 6: Same as Sample 5 of Experimental Example 1.

Sample 7: Same as Sample 6 of Experimental Example 1.

Sample 8: Same as Sample 7 of Experimental Example 1.

2) Testing method

The above samples were added to sterilized 1% bacto peptone (product of Difco Co.) to final concentrations of 200, 100, 50, 25, 12, 6, and 3 μg/mL, *Escherichia coli* 0111 or *Escherichia coli* IID-861 in logarithmic growth period added to 10^6 cells/mL of the culture, and incubated at 37°C for 17 h. The minimum concentration at which bacteria growth is not noted visually is given as the minimum inhibitory concentration (hereafter referred to as MIC) of the sample.

3) Test results

The test results are given in Table 2. As shown clearly in Table 2, Samples 2-8 showed MIC comparable to Sample 1 or less than 1/2, indicating strong antimicrobial effects. When tested with other peptide derivatives, nearly similar results were obtained.

[0041]

Table 2

1 ペプチドの種類	MIC (μg/ml)	
	E. coli 0111	E. coli IID-861
2 試料 1	12	6
試料 2	6	≤3
試料 3	6	≤3
試料 4	12	6
試料 5	12	6
試料 6	12	6
試料 7	6	≤3
試料 8	6	≤3

Key: 1 Type of peptide
2 Sample

[0042]

<u>Experimental Example 3</u>

This test is carried out for resistance of various compounds against protease.

1) Preparation of samples

Following three samples were prepared.

Sample 1: Same as Sample 1 of Experimental Example 1.

Sample 2: Peptide derivative of the Structure 10 below prepared by the method described in Application Example 10 by amidation of the terminal carboxy group, with the amino acid second from the amino terminal group being in D form and all other amino acids being in L form.

[0043]

[Structure 10]

Arg Arg Trp Gln Trp Arg-NH_2

[0044]

Sample 3: Same as sample 3 of Experimental Example 2

2) Test method

① Treatment of peptide derivatives by trypsin

The sample and trypsin (type IX, product of Sigma Co.) were added to 100 mM Tris-HCl buffer (pH 8.1) to final concentrations of 900 and 50 μg/mL, respectively, followed by incubation at 37°C for 1 h and heat treatment at 80°C for 10 min for trypsin deactivation.

② Derivation of trichophyton-produced protease

Trichophyton mentagrophytes TIMM-1189 was incubated in a culture (2% keratin powder, 1.2% yeast carbon base (product of Difco Co.), 0.005% inositol, 0.001% thiamine, 0.001% pyridoxine) by shaking at 27°C for 10 days. After confirming the derivation of a protease with bovine albumin as the substrate, the mixture was filtered to obtain a trichophyton-produced protease solution.

③ Treatment of peptide derivatives by the trichophyton-produced protease solution

Samples were added to the trichophyton-produced protease solution diluted two-fold to final concentration of 500 μg/mL, followed by incubation at 37°C for 1 h, then heat treatment at 80°C for 10 min for deactivation of the trichophyton-produced protease.

④ Measurement of antimicrobial activity of the peptide derivatives treated with protease

The antimicrobial properties of the protease-treated peptide derivatives against *Escherichia coli* 0111 were measured similarly by the method of Experimental Example 2.

3) Test results

The test results are given in Table 3. As clearly shown in Table 3, Sample 1 was completely deactivated by trypsin and the trichophyton-produced protease, while Sample 2 showed some residual activity and Sample 3 showed complete retention of the activity even after protease treatment. When tested with other peptide derivatives, almost identical results were obtained.

[0045]

Table 3

1 ペプチドの種類	MIC (μg/ml)		
	2 未処理	3 トリプシン処理	4 白癬菌産生プロテアーゼ処理
5 試料 1	12	>200	>200
試料 2	12	50	100
試料 3	6	6	6

Key:
1 Type of peptide
2 Untreated
3 Trypsin treated
4 Trichophyton-produced protease treated
5 Sample

[0046]

Experimental Example 4

This test was carried out to examine acute toxicity of various peptide derivatives.

1) Animals used

Using both sexes of 6-week-old CD (SD) rats (purchased from Nippon SLC), they were randomly divided among sexes into four groups (five per group).

2) Testing method

The peptide derivative prepared by repeating the method of Application Example 1 was dissolved to 1000 and 2000 mg/kg body weight in injection water (product of Otsuka Seiyaku Co.) and a single forced feeding was made at a ratio of 4 mL/100 g body weight using a metal ball fitted needle for acute toxicity test.

3) Test results

No mortality was observed in the group fed with the peptide derivative to 1000 mg/kg body weight and 2000 mg/kg body weight. Accordingly, the LD_{50} of the peptide derivative was above 2000 mg/kg body weight, indicating very low toxicity. When tested with other peptide derivatives, the results are about the same.

Reference example

50 mg commercial bovine lactoferrin (product of Sigma Co.) were dissolved in 0.9 mL of purified water, followed by adjusting the pH to 2.5 with 0.1N hydrochloric acid, the addition of 1 mg swine pepsin (product of Sigma Co.), hydrolysis at 37°C for 6 h, addition of 0.1N sodium hydroxide to a pH of 7.0, enzyme deactivation by heating at 80°C for 10 min, cooling to room temperature, centrifugal separation at 15,000 rpm for 30 min to obtain a clear supernatant, high-performance liquid chromatography of 100 μL of the supernatant using TSK Gel ODS-120T (product of Toso Co.), elution with 20% acetonitrile containing 0.05% TFA (trifluoroacetic acid) at a [flow] rate of 0.8 mL/min for 10 min after injection of the sample, gradient elution with 20-60% acetonitrile containing 0.05% of TFA for 30 min, collecting the fractions eluted during 24-25 min, drying the collected fractions in vacuo, dissolving the dried product to 2% (w/v) concentration in purified water, high-performance liquid chromatography again using TSK Gel ODS-120T (product of Toso Co.) eluting with 24% acetonitrile containing 0.05% TFA at a rate of 0.8 mL/min for 10 min after sample injection, gradient elution with 24-32% acetonitrile containing 0.05% TFA for 30 min, collecting the fractions eluted during 33.5-35.5 min, repeating this process 25 times, and vacuum drying of the collected fractions to obtain about 1.5 mg peptide.

[0047]

The above lactoferrin peptide was hydrolyzed by 6N hydrochloric acid and analyzed for amino acid composition by a conventional manner using an amino acid analyzer. The same sample was subjected to Edman degradation 25 times using a gas-phase sequencer (product of Applied Biosystems Co.) to determine the sequence of the 25 amino acid moieties. The presence of disulfide bond was confirmed by the disulfide bond analysis method (Analytical Chemistry, Vol. 67, p. 493, 1975) using DTNB (5,5-dithiobis(2-nitrobenzoic acid)).

[0048]

As a result, it was confirmed that this peptide comprises 25 amino acid moieties, the number 3 and number 20 cysteine moieties are disulfide bonded, and the N-terminal side of the number 3 cysteine is bonded to 2 amino acid moieties and the C-terminal side of the number 20

cysteine moiety is bonded to 5 amino acids to form an amino acid sequence described in sequence number 4.

[0049]

Next, the present invention is explained in further detail with examples. However, the present invention is not limited to such examples.

[0050]

Application Example 1

A peptide derivative was prepared as shown below according to solid-phase peptide synthesis method by Shepherd, et al.: Journal of Chemical Society Perkin I, p. 538, 1981, using a peptide synthesizer (LKB Biolynx 4170, product of Pharmacia LKB Biotechnology Co.).

[0051]

Using 1 g of a solid resin for C-terminal amide peptide synthesis (NovaSyn KR 0.1 mEq, product of Cal Biochem-Nova Biochem Co.) and the synthesis program of the above peptide synthesizer, the protective group removal reaction and condensation reaction are repeated for peptide chain extension. Namely, by 20% DMF, Fmoc group is cleaved and removed, followed by washing with DMF, reaction with 0.5 mmol each of Fmoc-amino acid active ester/N-hydroxybenzotriazole (hereafter referred to as HOBt), washing with DMF, and repeating the process. After the condensation, if needed, Kyser [transliteration] test is carried out to confirm the completion of coupling, then proceeds to the next step. The amino acids used are Fmoc-Lys(Boc)-Opfp, Fmoc-Trp(Boc)-Opfp, Fmoc-Gln-Opfp, Fmoc-Met-Opfp (all products of Cal Biochem-Nova Biochem Co.) in 0.5 mmol cartridges. After completion of the peptide chain extension reaction, the Fmoc group is cleaved by 20% piperidine/DMF, followed by washing with DMF then acetylation by 10% acetic anhydride/DMF. After confirmation of completion of the acetylation by the Kyser test, the resin is then washed well with DMF, tert-pentyl alcohol, acetic acid tert-pentyl alcohol, DMF, then diethyl ether and dried in vacuo.

[0052]

The above protected peptide resin (571 mg) was stirred with 1.0 mL ethanedithiol, 200 μL m-cresol and 2.4 mL thioanisole in an argon stream at room temperature for 15 min, further stirred for 10 min under ice cooling, stirred with 15 mL trifluoroacetic acid for 10 min, then with 2.6 mL trimethylsilyl bromide and further stirred for 50 min. The resin is filtered out using a glass filter, and the filtrate is concentrated in vacuo immediately. The residue is treated with cold diethyl ether to form a white peptide powder which is then separated by centrifugal

separation (2500 rpm, 5 min), freed from the supernatant, stirred with cold diethyl ether again and subjected to centrifugal separation. This separation process is repeated four times. The peptide precipitate is dried in vacuo, dissolved in water and lyophilized to obtain 62.5 mg of a crude peptide.

[0053]

The crude peptide is dissolved in water and centrifuged (15,000 rpm, 5 min). The supernatant is filtered through a 0.45 mm filter, and the peptide is purified by high-performance liquid chromatography (HPLC). The HPLC is done using Gariba PU-986 high-pressure gradient system (product of Nippon Bunko Co.) and reverse-phase column Lichrospher 100RP-18(e) 250 x 10 mm (product of Merck Co.) Elution is done with solution A of 0.1% TFA/water and solution B of 80% acetonitrile/solution A, with a solution A to solution B linear concentration gradient. The chromatogram almost has a single peak. The corresponding fraction is separated repeatedly and lyophilized to obtain 28.5 mg of a purified peptide.

[0054]

The purified product is confirmed to be a single compound by HPLC analysis using an analytical column (Lichrospher 100RP-18(e) 250 x 4.6 mm, product of Merck Co.). The purified peptide structure is confirmed by conventional amino acid analysis, amino acid sequence analysis, elemental analysis, mass analysis, etc., to be a peptide derivative shown by the Structure 11 below:

[0055]

[Structure 11]

CH_3CO-Lys Lys Trp Gln Trp Lys Met Lys Lys-NH_2

[0056]

<u>Application Example 2</u>

A peptide derivative was prepared as shown below by repeating the steps of Application Example 1 using a succinimide ester for introducing an acyl group. Namely, 2.8 g stearic acid ($CH_3(CH_2)_{16}COOH$) was dissolved in dichloromethane, treated with a solution of 1.2 g N-hydroxysuccinimide in DMF and a solution of 2.3 g dicyclohexylcarbodiimide in dichloromethane, stirred at room temperature for 5 h, monitoring the progress of the reaction by thin-layer chromatography to confirm the completion of the reaction. After filtering out the byproduct, dicyclohexylurea, the reaction mixture was concentrated in vacuo, and the resulting residue was mixed with diethyl ether and centrifuged (2500 rpm, 5 min). The supernatant was

discarded, and the residue was mixed with fresh diethyl ether, repeating the centrifugal separation twice. The product was filtered out using a glass filter and dried in vacuo to obtain 2.0 g stearic acid succinimide ester. The protected peptide resin (N-terminal amino group free, 0.1 mmol scale) synthesized by peptide synthesizer was mixed with a solution of 382 mg of the stearic acid succinimide ester in DMF and allowed to react at room temperature for 5 h, confirming the complete acylation by the Kyser test to obtain 1.13 g acylated protected peptide resin. The crude peptide yield after deprotection and de-resination was 181 mg. About 98 mg purified peptide derivative represented by Structure 12 shown below was obtained.

[0057]

[Structure 12]

Acyl(C18)-Arg Arg Trp Gln Trp Arg Met Lys Lys-NH_2

[0058]

Application Example 3

Application Example 1 was repeated using a condensing agent for acyl group introduction. A solution of 154 mg palmitic acid ($CH_3(CH_2)_{14}COOH$), 228 mg HBTU (2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate) and 92 mg HOBt in DMF was treated with 105 μL DIEA (diisopropylethylamine), added to a protected peptide resin (N-terminal amino group free, 60 μmol scale) synthesized by a peptide synthesizer, stirred at room temperature for 1 h, confirming complete acylation by the Kyser test to obtain 476 mg acylated protected peptide resin. After deprotection and de-resination, the crude peptide yield was 103 g. About 46 mg of a purified peptide derivative represented by the Structure 13 was obtained.

[0059]

[Structure 13]

Acyl(C16)-Arg Arg Trp Gln Trp Arg Met Lys Lys-NH_2

[0060]

Application Example 4

Application Example 3 was repeated to obtain 444 mg acylated protected peptide resin. After deprotection and de-resinification, the crude peptide yield was 92 mg. About 47 mg of a purified peptide derivative represented by Structure 14 was obtained.

[0061]

[Structure 14]

Acyl(C14)-Arg Arg Trp Gln Trp Arg Met Lys Lys-NH_2

[0062]

Application Example 5

Application Example 3 was repeated to obtain 493 mg acylated protected peptide resin. After deprotection and de-resinification, the crude peptide yield was 101 mg. About 53 mg of a purified peptide derivative represented by the Structure 15 was obtained.

[0063]

[Structure 15]

Acyl(C10)-Arg Arg Trp Gln Trp Arg Met Lys Lys-NH_2

[0064]

Application Example 6

Application Example 3 was repeated to obtain 493 mg acylated protected peptide resin. After deprotection and de-resinification, the crude peptide yield was 105 mg. About 57 mg of a purified peptide derivative represented by Structure 16 was obtained.

[0065]

[Structure 16]

Acyl(C6)-Arg Arg Trp Gln Trp Arg Met Lys Lys-NH_2

[0066]

Application Example 7

Application Example 1 was repeated using a solid resin Tanta Gel-NH_2 (polyethylene glycol (hereafter referred to as PEG) spacer part molecular weight: 3000, product of Shimazu Seisakusho Co.) to obtain 484 mg protected peptide resin. After deprotection and de-resinification, the crude peptide yield was 311 mg. About 132 mg of a purified peptide derivative represented by Structure 17 was obtained.

[0067]

[Structure 17]

CH_3CO-Arg Arg Trp Gln Trp Arg Met Lys Lys-PEG

[0068]

Application Example 8

Application Example 1 was repeated to obtain 562 mg acylated protected peptide resin. After deprotection and de-resinification, the crude peptide yield was 62 mg. About 29 mg of a purified peptide derivative represented by the Structure 18 was obtained.

[0069]

[Structure 18]

Lys Lys Trp Gln Trp Lys Met Lys Lys-NH_2

[0070]

Application Example 9

Application Example 1 was repeated with peptide bond formation reaction by HBTU/HOBt condensation agent. Thus, the amino acid introduction of the peptide synthesizer was performed manually. The amino acids used were Fmoc-D-Arg(Mtr)-OH, Fmoc-D-Lys(Boc)-OH, Fmoc-D-Trp(Boc)-OH, Fmoc-D-Gln-OH and Fmoc-D-Met-OH (all products of Watanabe Kagaku Kogyo Co.) to obtain 502 mg of protected peptide resin. After deprotection and de-resinification, the crude peptide yield was 57 mg. About 32 mg of a purified peptide derivative represented by the Structure 19 was obtained.

[0071]

[Structure 19]

CH_3CO-Arg Arg Trp Gln Trp Arg Met Lys Lys-NH_2

[0072]

Application Example 10

Application Example 1 was repeated, but the introduction of the amino acid Arg at number 2 from the N terminal is done by the method of Application Example 9 using HBTU/HOBt condensation agent. The amino acids used were Fmoc-Arg(Mtr)-Opfp, Fmoc-Trp(Boc)-Opfp, Fmoc-Gln-Opfp (all products of Cal Biochem-Novo Biochem Co.). Fmoc-D-Arg(Mtr)-OH (product of Watanabe Kagaku Kogyo Co.) was used for the number 2 amino acid from the N terminal. The yield of the protected peptide resin was 188 mg. After deprotection and de-resinification, the crude peptide yield was 14 mg. About 8 mg of a purified peptide derivative represented by the Structure 20 was obtained.

[0073]

[Structure 20]

Arg Arg Trp Gln Trp Arg-NH_2

[0074]

Application Example 11

Tablets of the following composition were prepared by a conventional manner.

Lactose	79.45 (%)
Peptide derivative of Application Example 2	0.55
Magnesium stearate	20.00

Application Example 12

An injectable agent of the following composition was prepared by a conventional manner.

[0075]

Peptide derivative of Application Example 9	1.20 (%)
Surfactant	8.00
Saline solution	90.80

Application Example 13

A chewing gum of the following composition was prepared by a conventional manner

[0076]

Gum base	25.00 (%)
Calcium carbonate	2.00
Fragrance	1.00
Peptide derivative of Application Example 1	0.10
Sorbitol powder	71.90

Application Example 14

A hand lotion of the following composition was prepared by a conventional manner.

[0077]

Carbowax 1500	9.00 (%)
Alcohol	4.00
Propylene glycol	47.00
Fragrance	0.50
Peptide derivative of Application Example 7	0.40
Distilled water	39.10

[0078]

Effects of the invention

As explained in detail above, the present invention provides antioxidants having excellent suppression action against active oxygen, free radicals, and lipid peroxidation, and antimicrobial agents having excellent antimicrobial action against microorganisms such as bacteria, fungi, etc. Such antioxidants and antimicrobial agents are extremely safe when used in drugs, food, etc.

[0079]

Sequence table

Sequence number: 1

Sequence length: 6

Sequence type: amino acid

Topology: linear

Sequence type: peptide

Sequence: Arg Arg Trp Gln Trp Arg

1 5

Sequence number: 2

Sequence length: 9

Sequence type: amino acid

Topology: linear

Sequence type: peptide

Sequence: Arg Arg Trp Gln Trp Arg Met Lys Lys

1 5

Sequence number: 3

Sequence length: 9

Sequence type: amino acid

Topology: linear

Sequence type: peptide

Sequence: Lys Lys Trp Gln Trp Lys Met Lys Lys

1 5

Sequence number: 4

Sequence length: 25

Sequence type: amino acid

Topology: linear

Sequence type: peptide

Sequence: Phe Lys Cys Arg Arg Trp Gln Trp Arg Met Lys Lys Leu Gly Ala Pro

1 5 10 15

Ser Ile Thr Cys Val Arg Arg Ala Phe

20 25